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Universal calibration of size-exclusion chromatography for proteins in guanidinium hydrochloride including the high-molecular-mass proteins titin and nebulin

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ABSTRACT

The chromatography of polypeptides in 6 *M* guanidinium hydrochloride (GuHCl) was studied with respect to the measurement of two ultra-large polypeptides, titin and nebulin. These proteins are integral constituents of the muscle structure, responsible for elasticity, and play an important role in muscle function. Both are outside the previously available range of calibration standards. This problem was circumvented by universal calibration in two different solvents, namely denaturing GuHCl conditions for the unknown polypeptides and buffered solutions of viruses under native assembly conditions. The accuracy of the approach was established. Two matrices were tested for their stability towards this solvent change without changing their calibration graphs. For Superose-6 the calibration changed by 10%. TSK-6000PW exhibited a congruent calibration graph for native and denaturing conditions. By extrapolation it was possible to estimate the chain molecular masses for nebulin to be 560 000 and that for T-II, the extractable form of titin, to be 2 000 000.

INTRODUCTION

The size-exclusion chromatography (SEC) of proteins under denaturing conditions is widely used to study polypeptide chain molecular masses. Sodium dodecul sulphate (SDS), urea and guanidinium hydrochloride (GuHCl) have been employed to this end [1]. GuHCl (6 *M*) under reducing conditions or with alkylated proteins seems to be the most successful [2–19]. GuHCl breaks multimeric enzymes apart, unfolds the proteins, binds to the peptide backbone and to aromatic residues [20] and induces an extended coiled conformation whose dimensions are virtually sequence independent if disulphide bonds are broken. Normally calibration of the chromatographic columns is done directly in terms of molecular mass with the aid of some reference peptides of known molecular mass. This strategy fails for large peptides for which no references exist. This paper is devoted to this case of ultra-large polypeptide chains. It attempts universal calibration valid for different solvent conditions. Native polymeric protein assemblies, which would dissociate and denature in GuHCl, were eluted at regular ionic strength and compared with denatured proteins in 6 *M* GuHCl on the same matrix. Two different matrices were tested.

Universal calibration of SEC in a broad sense is an attempt to define a unique parameter that accounts for the elution properties of all chemical substances and physical shapes. In a narrower sense it refers to the suggestion of Grubisic *et al.* [21] that this parameter is found in the hydro-

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dynamic volume, *viz.*, the cube of the viscosity radius, defined as [22]

$$R_\eta = \left(\frac{3}{10\pi N_{\rm L}} \cdot [\eta]M\right)^{1/3} = 0.0541\ ([\eta]M)^{1/3} \quad (1)$$

where R_η is the viscosity radius (nm), $[\eta]$ is the intrinsic viscosity (ml g^{-1}), M is the molecular mass and $N_{\rm L}$ is Avogadro's number. Note that eqn. 1 is frequently misprinted [14,23]. Judgement is still empirical and lacks a profound theory. However, calibration by viscosity radii yields the best correlations and is in this regard superior to all other known measures such as diffusional Stokes radius, radius of gyration and mean linear extension, which is sometimes erroneously called contour length, and also plain molecular mass [23–29]. At present only two systems, namely schizophyllan and DNA [23,28,29], are not represented by viscosity radii or any other of the mentioned measures. A future theory that truly accounts for all data therefore will have to define a physical shape function that coincides with intrinsic viscosity under most but not all circumstances.

Universal calibration is an idealized concept that excludes adsorptive effects which often trouble real systems. In choosing a proper solvent and matrix for a given solute, ideal conditions may be approached. Universal calibration demands that in a given eluent all solutes elute by a common criterion as long as adsorption is lacking. This criterion must further be independent of the matrix chosen except for the excluded case of adsorption. It does not require that two eluents given identical calibrations. In fact, the early matrices for biological applications all changed their pore structures with changes in pH and ionic strength. Thus each eluent required its own calibration. Only the recent high-performance matrices are rigid enough to withstand simple solvent changes. It was therefore possible to extend the theory of universal calibration to a comparison of different solvent conditions. All ionic effects are now accounted by a single theory [23]. This theory further predicts that different chemical classes follow a unique calibration only if solvation is explicitly accounted for. This concept has also been extended to

GuHCl solutions. It has been noted that the viscosity radius is the most appropriate measure [9,10,13,14,18]. Thus a common congruent calibration in terms of viscosity radii was constructed on the silicate TSK-SW matrix [10,14] and on Sepharose, a cross-linked agarose [18]. One study even included 8 M urea and SDS in their common congruent calibration [10]. On the other hand, Eriksson and Hjertén [16] found that a different cross-linked agarose is unstable towards chemical exposure to GuHCl and completely changes pore size.

According to theory, R_η and R_s are virtually identical for globular compact proteins. For spherical particles R_s is measured with greater precision that intrinsic viscosity and is thus substituted for R_η. For elongated shapes $R_\eta > R_s$, the exact value of which depends on quaternary structure and flexibility. For denatured coils R_η is greater than R_s typically by about 15% based on experimental figures. A simple theory predicts about a 30% difference [30]. Thus data for GuHCl conditions appear early on the basis of R_s. Horiike *et al.* [14] most explicitly demonstrated this fact chromatographically. They thus confirmed that congruent calibration fails for diffusional Stokes radii as a putative universal parameter. Excellent correlation is obtained, however, using viscosity radii. To complicate matters, native proteins show gradually decreasing elution volumes with increasing GuHCl concentration prior to denaturation [31–34]. One possible explanation calls for a premature elution of proteins in GuHCl and universal calibration fails even in terms of R_η.

Notwithstanding these difficulties, we were able to assign molecular masses of unknown polypeptides in 6 M GuHCl on TSK-6000PW by reference to native proteins and viruses and to calculate the chain molecular masses. This is a particularly intriguing task for titin, probably the largest single polypeptide chain every found. Titin, previously also called connectin, is a major component of the elastic filaments in sarcomeric muscles (for reviews see refs. 35–37). Direct solubilization of myofibrils by SDS yields a doublet on polyacrylamide gel electrophoresis (SDS-PAGE), T-I and T-II. In the sarcomer, the ends of the titin T-I molecules are attached to

the M band and the Z band, respectively, and titin keeps the acto-myosin filament system in register. It also provides an elastic component beyond the acto-myosin system. Titin I–II is a proteolytic derivative of the parent T-I molecule, as shown by monoclonal antibodies, and it stretches from the M band to very close to the Z line of the sarcomer [38]. It is the only titin species extractable under native conditions in a variety of protocols [39–42] and will be characterized here. Molecular mass estimates based on SDS-PAGE, chromatography, ultracentrifugation and electron microscopy range from 800 000 to 2 800 000 [17,39–45] (see Table I). With our method we obtain a molecular mass of 2 000 000 for titin T-II. Titin T-I is estimated to be 200 000 [44] to (more likely) 700 000 [39,46] larger than the 2 000 000 M_r titin T-II. Native titin T-II is a mixture of aggregates that separates into two peaks on chromatography on TSK-6000PW. Titin T-II$_A$, still a broad inhomogeneous band, contains two M-line associated proteins of M_r 165 000 and 190 000 by SDS-PAGE on overloaded gels. They are visualized by electron microscopy as globular beads on one end of the titin molecule. They help to associate titin molecules and several, mostly two, titin tails protrude from these knobs as judged by electron microscopy [42]. These proteins dissociate in 4 M GuHCl and are removed in SEC by virtue of their size. The titin band in 4 M GuHCl lacks these associated proteins as judged by SDS-PAGE. The present study identifies titin T-II$_B$ as the native monomer. Mild trypsin treatment of either T-II$_A$ or T-II$_B$ removes the mentioned terminal head domain (knobs) and causes T-II$_A$ to dissociate. The resulting tail domain elutes identically regardless of whether it originates from T-II$_A$ or T-II$_B$ and lacks the associated proteins. It elutes about 0.1 ml after the native T-II$_B$ band, in line with the observation that inadvertent proteolysis enlarges the apparent T-II$_B$ peak. This small retention difference implies that the head domain cannot be much larger than M_r ca. 50 000. Titin T-II and the tail are not distinguished by SDS-PAGE.

As a second large polypeptide, we studied another myofibrillar protein, nebulin, which is only present in skeletal muscle. Much less is known about this protein, with a molecular mass

TABLE I

DATA ON SIZE AND SHAPE OF TITIN

Type of titin	Method[a]	M_r (×1000)	s (S)	R_{SEC} (nm)	Ref.
Chicken, native T-II$_A$/T-II$_B$ mixture[b]	Sedimentation equilibrium	2700	17		39
Chicken, native T-II$_A$	SEC		27–57	75–95	42
Chicken, native T-II$_B$	SEC		12.5	48	42
Native T-II$_B$ enriched mixture	–		13		40
Native T-II$_B$	STEM[c]	2400 ± 500			45
Rabbit, denatured T-II	SDS-PAGE based on glutaraldehyde-cross-linked myosin-H	1200			44
Chicken, denatured T-II	SDS-PAGE based on bismaleimide-cross-linked myosin-H	2100			39
Denatured T-II	SEC in 6 M GuHCl	800–1600			17
Rabbit, denatured T-I/II mixture	Sedimentation equilibrium in 6 M GuHCl	2400–2600[d]	13[e]		97
Chicken, denatured T-II	SEC in 6 M GuHCl	2000		53	This study

[a] Methods other than sedimentation velocity.
[b] Judged to be a mixture by comparison with other entries in this table.
[c] Scanning transmission electron microscopy.
[d] Based on partial specific volume $v = 0.727$.
[e] Authors report 4.15 S, which apparently was not corrected to unit viscosity.

232

R. Nave et al. / J. Chromatogr. A 654 (1993) 229-246

in the range 500 000–900 000 calculated on the basis of its mobility in gel electrophoresis [36,39,43,46,47]. Resistant to extraction under native conditions, its size and shape are not known. However, it has been suggested that nebulin could form a fourth filament system in skeletal muscle [47–49] and may be the length regulator of thin filaments [46]. In the sarcomer, nebulin is primarily located within the I-band. Here we estimate a molecular mass of 560 000 for the monomer unit by SEC in GuHCl.

EXPERIMENTAL

Size-exclusion chromatography

The chromatographic instrumentation has been described in detail previously [24,25]. The columns consisted of a Superose-6 cross-linked agarose matrix in a 30-cm glass cartridge (Pharmacia–LKB) and a TSK6000-PW 60-cm stainless-steel column (Toso Haas–Pharmacia). Both columns have been described previously [23–26]. Flow-rates were less than 0.3 ml min^{-1}, corresponding to shear rates below 300 s^{-1} for the present experimental set-up. Absence of shear degradation was verified by SDS-PAGE (for a general discussion of shear degradation, see ref. 50). Elution was monitored by measuring the absorbance at 280 nm. Sample concentrations were kept to a minimum, e.g., not exceeding 0.2 mg ml^{-1} for titin, to avoid concentration artifacts. The 6 M GuHCl eluent contained 0.4 mM β-mercaptoethanol, 50 mM Tris–HCl (pH 7.3) and 1 mM EDTA.

Materials

Most of the samples used for calibration have been described previously [24]. Briefly, we used albumin from bovine serum (Sigma A8531), alcohol dehydrogenase from yeast (Sigma A8656), aldolase from rabbit muscle (Sigma A6253), alkaline phosphatase from yeast (Sigma P9761), apoferritin from horse spleen (Sigma A3660), β-galactosidase from Escherichia coli (Sigma G8511, G6008), carbonic anhydrase from bovine erythrocytes (Sigma C7025), catalase from bovine liver (Sigma C100, Pharmacia 17044101), cytochrome c from horse heart

(Sigma C7150), bovine gamma-immunoglobulin (Sigma I5506, BioRad 1511901), myoglobin from horse heart (Sigma M1882, Bio-Rad 1511901), ovalbumin from hen egg white (Pharmacia 17044201, Bio-Rad 1511901), ovomucoic from hen egg white (Sigma T2011), phosphorylase B from rabbit muscle (Boehringer 108570), thyroglobulin from pig thyroid (Sigma T1126, Bio-Rad 1511901), human transferrin (Sigma T2252), bovine α-trypsin, urease from jack beans (Sigma U7752), ATP (grade II, Sigma A3377) and vitamin B_{12} (Bio-Rad 1511901). Tomato bushy stunt virus (TBSV) and turnip yellow mosaic virus (TYMV) were kindly provided by J. Witz, Strasbourg; bacteriophages Qβ and MS2 were a gift from C. Biebricher, Göttingen; rabbit muscle tropomyosin was a gift from A. Wegner, Bochum; chicken muscle actin was a gift from D.O. Fürst, Göttingen; human erythrocyte spectrin was provided by S. Eber, Göttingen, and the haemolymph of Eurypelma californicum obtained from H.J. Schneider, Munich, was used to study haemocyanin under oxidative conditions; bovine brain clathrin was a gift from E. Ungewickel, Munich; and pig neurofilament protein NF200 was a gift from N. Geisler, Göttingen. Tobacco mosaic virus (TMV) was prepared as described previously [51]. Titin, nebulin and myosin were prepared as follows.

Preparation of titin and nebulin

Native titin T-II was purified from chicken pectoralis muscle as described previously [42]. The final step was SEC through Superose-6. This material was then dialysed to buffered 6 M GuHCl (see above) and analysed. In addition, native titin T-II was fractionated in high-salt buffer [500 mM KCl, 50 mM Tris–HCl (pH 7.9), 2 mM EGTA, 1 mM β-mercaptoethanol] as described in detail previously [42]. The two bands T-II$_A$ and T-II$_B$ were separately dialysed to 4 M GuHCl and analysed.

Enriched fractions of nebulin were obtained by extraction of myofibrils from chicken breast muscle with 0.6 M KCl and 1 M KI to remove most of the actin, myosin and titin as described [38]. These "high-salt extracted myofibrils" were subsequently solubilized in 6 M GuHCl [50 mM

Tris–HCl (pH 8.6), 1 mM EDTA, 10 mM β-mercaptoethanol] and repeatedly applied to a Superose-6 SEC column equilibrated in 6 M GuHCl [20 mM Tris–HCl (pH 7.3), 1 mM EDTA, 0.4 mM β-mercaptoethanol]. Nebulin essentially eluted in the void fraction and all smaller peptides were removed. An amount of 100 μg of nebulin could be obtained per run, requiring 30 min. Fractions from repetitive runs containing nebulin were pooled. The entire preparation could be accomplished within 1 day, which minimizes the risk of proteolytic breakdown.

Titin and nebulin were detected by gel electrophoresis and immunoblotting with monoclonal antibodies as described [38].

Cross-linking of myosin

During the preparation of titin, fractions enriched in myosin were obtained [42]. A subsequent SEC step on Superose-6 in high-salt buffer [0.6 M NaCl, 50 mM Tris–HCl (pH 7.9), 1 mM EDTA] yielded pure myosin heavy chains. For chemical cross-linking, myosin was dialysed against a different high-salt buffer [0.6 M NaCl, 25 mM sodium borate buffer (pH 9.0)] and then treated with p-N,N'-phenylenedimaleimide (Sigma P3396) at room temperature [52]. The molar ratio of the reagent to myosin was 10:1 and the reaction was stopped after 30 min by adding an excess of β-mercaptoethanol. The extent of cross-linking was monitored by SDS-PAGE.

RESULTS

Reference data for denaturing conditions

It was demonstrated in the past that reduced proteins denatured in 6 M GuHCl are well characterized by a Mark–Houwink relationship establishing a correlation between molecular mass and intrinsic viscosity or, for that matter, viscosity radii R_η. Surprised by the small but notable lack of universal calibration mentioned in the Introduction, we decided to review all available primary reference data and recalculate the Mark–Houwink relationship. This equation is subsequently applied in Table IV to calculate calibration data for the materials actually studied

and its validity is crucial. Table II lists all molecular masses, intrinsic viscosities and corresponding viscosity radii found in the literature for reduced proteins in 6 M GuHCl. Most of them were determined in Tanford's laboratory. Two observations (glucose oxidase [53], ovomucoid [54]) were omitted from the table and subsequent calculation because their data seemed in error [9]. Older data for myosin [55–57] were rejected. A former value for albumin [13,58] has been adjusted by new measurements [19]. Data obtained in 5 M GuHCl (glyceraldehyde-3-phosphate dehydrogenase [59], thyroglobulin [60], tropomyosin [61]) and 7.5 M GuHCl (paramyosin [1,22,61]) have not been considered. For cytochrome c the molecular mass for the apo-enzyme is listed in Table II. The haeme group (an extra 617 in molecular mass) is covalently attached and makes cytochrome c an unreliable reference. If available, molecular masses have been calculated from the protein's sequence. This explains differences between Table II and previous compilations. The data are shown in Fig. 1. The resulting correlation between viscosity radius (nm) and molecular mass is

$$R_\eta = 1.73_5 \cdot 10^{-2} M^{0.552} \qquad (2)$$

with a correlation coefficient of $\bar{r} = 0.9997$. The mean accuracy, defined in the section *Accuracy of measurement* below, is $1.9 \pm 1.2\%$. This is slightly different from the coefficients normally quoted, $1.676 \cdot 10^{-2}$ and 0.555, respectively [22], but it makes no difference over the applicable data range. In conclusion, reference viscosity radii seem to be reliable.

Reference data for non-denaturing conditions

The proper definition of R_{SEC} as the ultimate ratio of universal calibration (see Discussion) is still unsettled and R_η values are currently substituted for it. For nearly spherical objects this reduces to the equality $R_\eta = R_s$. For native proteins we used the same hydrodynamic constants as previously [24]. The reference values for apoferritin and urease are debated and thus uncertain in the range 6.1–6.6 nm (discussed in ref. 62). As can be seen from the data in Table

234

R. Nave et al. / J. Chromatogr. A 654 (1993) 229–246

TABLE II

INTRINSIC VISCOSITY REFERENCE DATA IN 6 M GuHCl

Name	Origin	M_r [a]	$[\eta]$ (ml g^{-1})	R_η (nm)	Ref.
Albumin	Bovine serum	66 463	50.1	8.08	19
Aldolase	Rabbit muscle	39 211	35.3	6.03	58
Carbonic anhydrase		29 000	29.6	5.14	22
α-Chymotrypsinogen A	Bovine pancreas	25 666	26.8	4.78	58
Cytochrome c	Horse heart	11 702	14.4	2.99	4
Haemoglobin ($\alpha + \beta$) (apo)	Human	15 500	18.9	3.60	58
Insulin (A + B)	Bovine	2870	6.1	1.40	58
Lactate dehydrogenase	Bovine heart	36 500	32.4	5.72	98
β-Lactoglobulin		18 400	22.8	4.05	58
Lysozyme	Chicken	14 313	17.1	3.38	22, 99
Myoglobin (apo)	Horse	16 950	20.9	3.83	58
Myosin H-chain		224 000	107.5	15.62	22
Ovalbumin	Hen egg white	44 310[c]	34.6	6.22	98
Pepsinogen		40 000	31.5	5.84	58
ATP phosphoribosyltransferase	*Salmonella typhimurium*	33 211	31.9	5.51	100
Ribonuclease A	Bovine	13 690	16.6	3.30	58
Surface antigen 51A protein	*Paramecium aurelia*	301 500	133.4	18.54	101
Surface antigen 51B protein	*Paramecium aurelia*	260 000	115.2	16.80	101
Surface antigen 51D protein	*Paramecium aurelia*	270 000	123.0	17.39	101
Transferrin	Human	82 555[d]	50.8	8.72	4, 102
Uromodulin[b]	Human	86 000[e]	52.9	8.96	103

[a] Molecular masses are based on sequence data whenever they were available in the SWISSPROT data bank.
[b] Formerly called Tamm–Horsfall urinary glycoprotein.
[c] Based on M_r 1560 carbohydrate [104].
[d] Based on M_r 6200 carbohydrate [104].
[e] Based on 28% carbohydrate [9].

III, the assignment is ambiguous and their order of elution depends on the column used. The issue could not yet be settled. The Stokes radius for α-trypsin was taken from the diffusion coefficient [63]. The value for 27S-thyroglobulin, *i.e.*, dimers of the primary native species, is taken from interpolation of chromatographic elution on two different matrices (Superose-6 and TSK-5000PW) and should properly be called an R_{SEC} value only. The size of the spherical virus also is well established and previous values are used [24]. The calibration data for TMV are reviewed and insignificantly revised from previous choices as follows.

Depending on conditions, solutions of TMV easily aggregate or disassemble, which historical-ly has resulted in tremendous efforts to establish proper physical constants. The now settled electron microscopic (EM) length of the virus monomer is 300 nm [64,65]; values of 280 nm [66,67] and 270 nm [68] were reported previously. EM specimen preparation may break the virus particles on drying and part of the size variation may be an artifact without correspondence to solution properties [69]. In line with this reasoning is a 270-nm preparation that was found to be infectious [70] and its solution may well have contained proper 300-nm particles. Further, the intrinsic viscosity of 270-nm preparations is identical with if not larger than that of well defined 300-nm preparations [68,71].
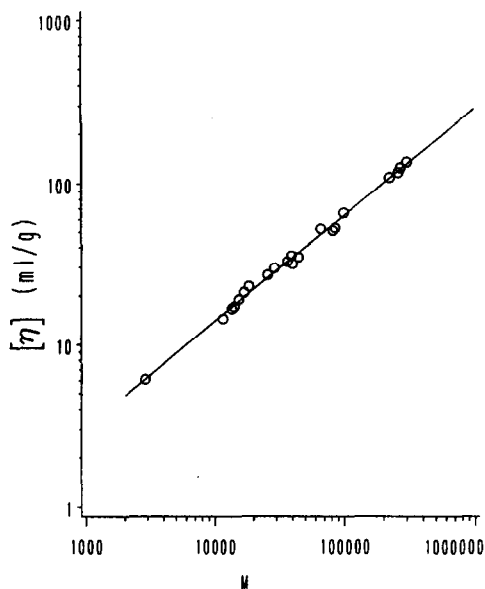
Based on a 300-nm EM length, the X-ray

Fig. 1. Mark–Houwink relationship for proteins in 6 M GuHCl based on all available data given in Table II. The resulting correlation of intrinsic viscosity (ml g^{-1}) and molecular mass is $[\eta] = 3.16 \cdot 10^{-2}\ M^{0.66}$.

structure and the protein sequence, the molecular mass of the virus monomer is $39.4 \cdot 10^6$. This agrees well with light-scattering data of $39.0 \cdot 10^6$ [71] and $40.0 \cdot 10^6$ [72] and with values calculated from sedimentation and diffusion. At infinite dilution monomers sediment with $s_{20,w}^0$ values (in Svedberg units) of 185 S [68,73,74], 187 S [68], 188 S [71] or 198 S [69,75], albeit part of the latter data are equally represented by 190 S [69]. Sedimentation coefficients of rod-shaped objects primarily depend on the diameter and are insensitive to moderate length heterogeneity. The following diffusion coefficients, D_{20}^0, have been reported: $5.3 \cdot 10^{-8}$ cm^2 s^{-1} for a 270-nm preparation [68], $5.0 \cdot 10^{-8}$ cm^2 s^{-1} derived from the spreading sedimentation boundary extrapolated to the meniscus position to eliminate the effect of boundary sharpening [76], $4 \cdot 10^{-8}$ cm^2 s^{-1} as a crude determination [74], $4.4 \cdot 10^{-8}$ cm^2 s^{-1} (if the corresponding s-value truly were 198 S, a value of $4.6 \cdot 10^{-8}$ cm^2 s^{-1} would match the molecular mass better) [69] and $4.75 \cdot 10^{-8}$ cm^2 s^{-1} as the latest value [75]. The diffusion constant is very sensitive to length heterogeneity and measurements are less reliable than those for sedimentation coefficients. Stokes radii for TMV

monomers based on diffusion measurements fall into the range 45–50 nm; larger values are undoubtedly aggregates and smaller ones fragments. Partial specific volumes (v) have been given as 0.727, 0.730 and 0.743 ml g^{-1} [68,75]. Proper Stokes radii may also be calculated from molecular mass, s value and partial specific volume. Compared with diffusion measurements, the reproducibility of measured intrinsic viscosity is excellent even though it also depends heavily on the length of the rods. Values of $[\eta] = 36.7$ ml g^{-1} [71] for a 300-nm preparation [65], 39.0 ml g^{-1} for a 270-nm preparation [68], 36.5 ml g^{-1} [74], 37.0 ml g^{-1} [77] and 32.0 ml g^{-1} [78] have been reported. Together with the molecular mass the viscosity radius is $R_\eta = 62$ nm. Eqn. 3 in ref. 24 uniquely relates R_η and R_s and together with sedimentation coefficients sets the most likely value as $R_s = 49$ nm. This yields excellent agreement with the EM length using eqn. 2 of ref. 24. (Note that earlier equations based on ellipsoidal shapes give wrong results [24,71].) All the above parameters are interrelated by well established hydrodynamic relationships [24] and may be cross-checked. The following is an optimized balanced data set for TMV virus monomers: $M = 39.4 \cdot 10^6$, $s_{20,w}^0 = 191$ S, $D_{20}^0 = 4.4 \cdot 10^{-8}$ cm^2 s^{-1}, $[\eta] = 37$ ml g^{-1}, $v = 0.730$ ml g^{-1}, hydration $H = 0.7$ g H$_2$O g^{-1}, frictional ratio $f/f_0 = 2.2$, length $L = 300$ nm, 2130 subunits, $R_s = 49$ nm, $R_\eta = 62$ nm.

It is possible to prepare nearly pure preparations of virus monomers, but depending on solution conditions and age of the sample an additional sedimentation boundary may appear and physical parameters of such mixtures change. This extra boundary has a sedimentation coefficient of 220 S [73], 216 S [68] or 210 S [74], as would be expected for an axial dimer of the above-described monomeric virus particle. Disaggregation of virus proceeds in discrete steps owing to the preferential affinity of the RNA sequence to the coat protein [79] and yields what is called partially stripped viruses. The latter seem preferentially to induce aggregation [69] and a large fraction of the observed aggregates may therefore be shorter than proper dimers [68,80]. Like sedimentation, SEC also yields separate peaks but so far no preparative amounts

TABLE III

ELUTION VOLUMES OF NATIVE PROTEINS AND VIRUSES (NON-DENATURING CONDITIONS)

Sample	R_η [a] (nm)	V (ml) Superose-6			TSK-6000PW	
		$I = 100$ mM, pH = 8.0 (40 mM borate, 98 mM NaF)	$I = 214$ mM, pH = 7.0 (100 mM Na phosphate)	$I = 1214$ mM, pH = 7.0 (100 mM Na phosphate, 1000 mM NaCl)	$I = 60$ mM, pH = 8.0 (40 mM borate, 58 mM NaF)	$I = 202$ mM, pH = 6.86 (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM $EDTA$-Na_2, 179 mM NaCl)
TMV dimer	(117)				13.30	13.21
TMV	62	7.01 (void)			15.08	14.85
Spectrin, tetramer	21.8	8.37			18.17	
TBSV	17.2[b]	9.33			18.7	
TYMV	15.1[b]	10.03			19.11	19.12
Spectrin, dimer	14.1	10.15			19.21	19.28
Qβ	14.3[b]	10.59			19.17	
MS2	13.9[b]	10.59			19.37	19.63
Thyroglobulin 27S[c]	12.1[d]	11.22	11.42	11.45		
Haemocyanin	10.8[b]	11.78	12.18		19.80	19.84
Thyroglobulin 19S[c]	8.6[b]	12.98	13.26	13.21	20.07	20.22
β-Galactosidase	6.86[b]	14.33				
Urease	6.34[b]	14.55			20.78	
Apoferritin	6.06[b]	14.85			20.60	
Catalase	5.23[b]	16.17			20.90	
Immunoglobulin G	5.23[b]	16.18	15.96	16.15		
Alcohol dehydrogenase	4.55[b]	16.34				
Albumin, bovine serum	3.62[b]	16.72			21.16	
Alkaline phosphatase	3.30[b]	16.99			21.35	21.65
Ovalbumin	2.83[b]	17.65	17.91	17.85		
Ovomucoid	2.75[b]	17.45				
Carbonic anhydrase	2.01[b]	18.50				
α-Trypsin	1.95[b]		18.67			
Myoglobin	1.91[b]	18.89	18.69	18.74		
ATP	–	20.30				
Cytochrome c	1.63[b]	21.08			22.5	
Vitamin B_{12} (V_{TOT})	–	21.72	21.78	22.35	23.0	22.90

[a] For asymmetric particles true R_η values are listed.
[b] For known spherical particles the R_s value has been used instead of proper values of intrinsic viscosity.
[c] 19S is the major species of 670 000 molecular mass.
[d] R_{SEC} value (see text).

of pure "dimers" have been isolated for detailed studies. A value of $[\eta]_{dimer} = 140$ ml g^{-1} may be calculated from a Mark–Houwink relationship [23]. Intrinsic viscosity measurement of a mixture of 61% monomer and 39% dimers (80.7 ml g^{-1}) is slightly larger than expected for true 600 nm dimers, which contradicts the lower apparent size of EM images [68]. This leads to $R_\eta = 117$ (±3) nm, with the upper bound for true dimers and the lower bound for worst case fragments. A value of 115 nm was obtained previously from SEC by extrapolating the calibration graph beyond the TMV monomer peak [24]. Even though the term "dimer" may be partly misleading, one is confronted with a well reproducible preparation. Mixtures have also been studied by

quasi-elastic light scattering [81] and by diffusion [82], but their composition remained unrecognized. Occasionally distinctly larger aggregates have also been observed.

In terms of hydrodynamics, TMV is probably the best characterized high-molecular-mass biopolymer. Of the few similar-sized viruses that are known, it is the only one readily available for routine analysis. The behaviour of DNA, albeit extensively studied, is not yet sufficiently understood. A limited number of synthetic flexible chain polymers, albeit heterogeneous in size, might be used in a future study to secure the present calibration graph in the ultra-high molecular mass region. Any SEC study calibrated by TMV, other viruses or chain polymers obviously depends on the reliability of the reference data and its results are subject to any revision of those.

Attempted universal calibration for Superose-6

We started our study on Superose-6, which has a convenient size range to compare native proteins with those denatured in GuHCl. Table III specifies the viscosity radii of native reference compounds and presents elution volumes. Note that these values depend on the actual filling volume of the column and may also suffer from batch-to-bach variability in matrix production. Hence these elution volumes are representative for the brand but may may differ slightly for different fillings. Earlier investigations have shown that elution volumes do in any case depend strongly on the ionic strength [23,25,27]. For repulsive interaction R varies at low ionic strength with $I^{-1/2}$ and normally levels off around $I = 100–200$ mM ionic strength. Table III shows that for Superose-6 there is little further difference for 1 M salt. Elution volumes were the same with and without 0.4 mM β-mercaptoethanol in the presence of a helium purge. Note that there is a significant drift of vitamin B_{12} elution volumes with increasing salt which is not paralleled by proteins. Similar observations have been made previously on TSK-5000PW [25] and TSK-6000PW [23].

The eluent was then switched to 6 M GuHCl (see Experimental) and the proper set of reference standards was measured (Table IV). This type is normally the sole set studied for GuHCl

TABLE IV

ELUTION VOLUMES OF DENATURED PROTEINS IN 6 M GuHCl

Sample	M_r (×1000)	R_η (nm)		V (ml)	
		Table II	Calc. with eqn. 2	Superose-6	TSK-6000PW
Blue dextran	2000		~25[a]	7.04	
Myosin, cross-linked H-chain dimer	448		22.85	7.86	18.24
Spectrin (monomer)	230		15.81		18.75
Myosin H-chain	224	15.62		9.22	18.93
Clathrin	190		14.23	9.85	
Neurofilament NF200	114[b]		10.79	10.75	
β-Galactosidase	116.4		10.86	11.60	19.93
Phosphorylase B	97.2		9.83	11.82	20.05
Transferrin	82.6	8.72		12.50	
Albumin, bovine serum	66.5	8.19		12.80	20.76
Immunoglobulin G, H-chain	49.5		6.77	13.82	20.90
Actin	41.8		6.17	14.15	21.27
Aldolase	39.2	6.03		14.20	
Immunoglobulin G, L-chain	23.5		4.49	15.65	
ATP	–			19.80	

[a] Estimated from eqn. 1 with $[\eta] \approx 50$ [83].
[b] Estimated from a sequence mass of 109.587 plus up to 4.750 bound phosphate [105].

conditions. Again calibration data are listed, some directly from Table II and others calculated via eqn. 2 from the known molecular mass. Blue dextran, which is included in the list, is a heterogeneous polysaccharide [83] which is at least partially included in the separation range of Superose-6 and thus is unsuitable to measure the void volume. In addition to the reference data, Table IV also presents the elution volumes for the particular columns employed in this study. Fig. 2 presents the data for Superose-6. Both data sets give log–linear relationships between R_η and V (ml) in the first 60% of the separation range and this is the range recommended for analysis, but the calibrations differ by 10% in R_η or 0.5 ml in elution volume. This means that the protein coils appear to be 10% larger than predicted from their intrinsic viscosities in 6 M GuHCl. However, even blue dextran and, more
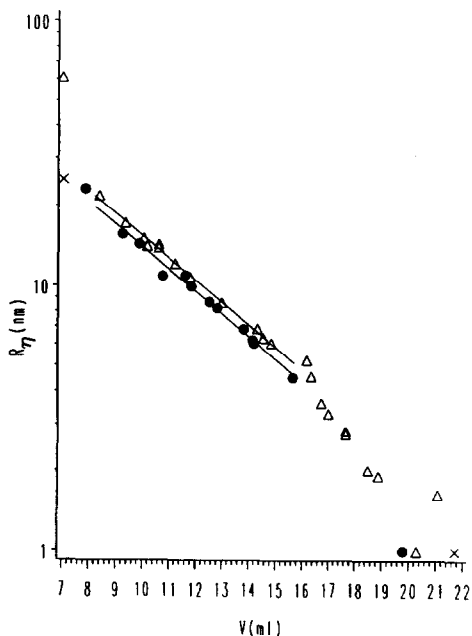
significantly, ATP are shifted by the same amount. Note that ATP, being charged, elutes differently from vitamin B_{12} and so will a number of small molecule markers. This illustrates the bias introduced in determining V_{TOT}.

The observed premature elution of proteins in GuHCl may have two reasons. The most likely is that the cross-linked agarose matrix changed conformation. Alternatively, elution simply would be premature and demonstrate once more that R_η is not the ultimate answer to universal calibration. However, if the latter were true, the effect had to be reproduced on a different matrix as universal calibration is assumed to be independent of the matrix.

Universal calibration for TSK-6000PW

We therefore repeated the measurement on a different matrix. The TSK-PW matrix is a hydrophilic polymer, albeit more hydrophobic than Superose, and TSK-6000PW is designed for the study of extremely large particles with viscosity radii above 10 nm up to at least 200 nm. Fig. 3 presents the data from Tables III and IV graphically and demonstrates a log–linear calibration in this size range which is only slightly dependent of ionic strength above 60 mM (up to at least 202 mM). This calibration is indirectly supported by a number of DNA standards that elute slightly differently but with a parallel calibration graph [23]. Some discrepancies do arise, however, in the last 40% of the separation range. This is the range after the bend of the calibration graph and is not recommended for analysis. In contrast to the range $R_\eta > 10$ nm there is a definite ionic strength dependence of these smaller native samples (difference between $I = 60$ and 202 mM) which predicts an even larger discrepancy of the GuHCl data ($I = 6\ M$), as observed. It is difficult to judge whether in addition the matrix is also changing for this size range and TSK-PW may be less resistant to exposure to GuHCl than one would wish. However, for those peptides having a viscosity radius in the linear working range of the column, a congruent universal calibration between denatured proteins in 6 M GuHCl and native viruses is obtained. TSK-PW thus appears to be usable for dual solvent experiments like TSK-SW [14] supposedly is. The different pat-
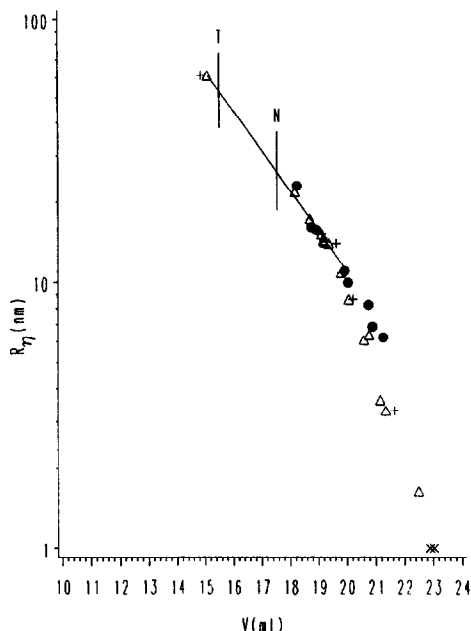

Fig. 2. Calibration of Superose-6. ($\triangle$) Native proteins, viruses and ATP and ($\times$) vitamin B_{12} at ionic strength $I = 100$ mM; data from Table III. ($\bullet$) Denatured polypeptides and ($\times$) blue dextran in 6 M GuHCl; data from Table IV. The log–linear calibration range extends from about 7.5 to 16 ml (solid lines); subsequently the calibration graph bends downwards. Cytochrome c is obviously somewhat adsorbed. Calibration of native and denatured conditions is virtually parallel but offset by about 0.5 ml in volume or 10% in R_η.

Fig. 3. Universal calibration of TSK-6000PW. Native proteins and viruses at ionic strength $I = (\triangle)$ 60 mM and $(+)$ 202 mM. $(\times)$ Vitamin B_{12} in both conditions; data from Table III. $(\bullet)$ Denatured polypeptides in 6 M GuHCl; data from Table IV. Elution volume measured for titin (T) is 15.5 ml and for nebulin (N) 17.54 ml. The calibration graph was fitted for the linear range of the $I = 60$ mM data but is valid for all data in the range 10–62 nm (solid line).

terns observed with Superose-6 and TSK-6000PW clearly evade a common mechanism other than to assume that Superose is indeed unstable to exposure to GuHCl.

Cross-linked myosin

The cross-linked dimer of myosin heavy chain eluted slightly later in GuHCl than expected from eqn. 2. Cross-linked proteins are branched polymers and not strictly comparable to linear-chain polypeptides. In principle, such a deviation is therefore to be expected. The actual size of cross-linked proteins depends critically on the extent of cross-linking and also on the type of cross-linker. For example, studies of fibrinogen have amply demonstrated that glutaraldehyde completely contracts the chain. Our preparation of myosin seems fairly well behaved, but a heavy reliance on cross-linked samples may lead to erroneous results.

Neurofilament protein

Apart from the cross-linked myosin, only the largest of the neurofilament triplet proteins, NF200, deviated substantially from calibration in GuHCl. Designated "200" for its apparent molecular mass by SDS-PAGE, it elutes from the GuHCl column like an M_r 140 000 protein [84]. Its true sequence of M_r 110 000 is only slightly larger than that of the medium neurofilament protein NF145. It is heavily phosphorylated, which may be the reason for abnormal behaviour in GuHCl. Still, chromatography in GuHCl gives a smaller error than SDS-PAGE.

Accuracy of measurement

The partition radius R_{SEC} is the radius read off the mutual calibration graph. The bias is due to uncertainties in the calibration data themselves and due to interfacial interactions that are only present in the porous matrix and not in the bulk solvent where calibration data had been measured. Table V lists R_{SEC} values for two sets of conditions. Note that the values depend on the matrix used. Comparison with similar reports by other workers further indicates that they are certainly biased by the choice of reference compounds on which they are based. The mean accuracy was computed by omitting one sample at a time and calculating the percentage deviation from the residual calibration graph. These percentage deviations were then averaged. The mean accuracy for the native Superose-6 data is $3.2 \pm 2.2\%$ for the linear calibration range (see Fig. 2). That for the GuHCl data on Superose-6 is $3.1 \pm 2.6\%$. This is slightly worse than the accuracy of eqn. 2 itself. For TSK-6000PW, native conditions at $I = 60$ mM, the corresponding figure is $2.4 \pm 1.7\%$ for the linear calibration range (see Fig. 3). Merging all data obtained on TSK-6000PW in the linear range (both native and GuHCl), the figure is $4.6 \pm 4.1\%$. The dual solvent method is therefore clearly less accurate than single solvent calibrations. However, even the accuracy of matching R_η by R_{SEC} in the single solvent method is significantly lower than the precision of measurement, i.e., the reproducibility in the determination of elution volumes, which is better than 1%. These discrepancies may in part be due to

TABLE V

PARTITION RADIUS R_{SEC} UNDER NON-DENATURING CONDITIONS

Sample	R_η (nm)	R_{SEC} (nm)	
		Superose-6 ($I = 100$ mM)	TSK-6000PW ($I = 60$ mM)
TMV	62		61.6
Spectrin, tetramer	21.8	21.0	20.8
TBSV	17.2	17.5	17.3
TYMV	15.1	15.3	15.0
Spectrin, dimer	14.1	14.9	14.5
Qβ	14.3	13.7	14.7
MS2	13.9	13.7	13.7
Thyroglobulin, dimer		12.1	
Haemocyanin	10.8	10.9	
Thyroglobin	8.6	8.67	
β-Galactosidase	6.86	6.69	
Urease	6.34	6.41	
Apoferritin	6.06	6.05	

inaccurate hydrodynamic reference data, but certainly also include minor systematic differences between R_η and R_{SEC} that have not previously been addressed.

Titin and nebulin

The theory presented and experimental data support the use of calibration graphs for TSK6000-PW outside the range accessible to monomeric polypeptides for both native and denatured GuHCl conditions. It is therefore straightforward to prepare unknown samples of titin or nebulin in 6 M GuHCl and to measure their elution volumes. Both native fractions of titin, T-II$_A$ and T-II$_B$, yielded identical elution volumes in GuHCl and lacked associated proteins as judged by SDS-PAGE. These elution volumes, 15.5 ml for titin T-II and 17.54 ml for nebulin, are then converted to viscosity radii via the universal calibration graph in Fig. 3. One obtains $R_\eta = 53$ nm for titin T-II and $R_\eta = 26$ nm for nebulin, each denatured in 6 M GuHCl. Subsequently the viscosity radius is converted into molecular mass via extrapolation of the Mark–Houwink relationship (eqn. 2), which strictly had only been obtained for the M_r range 2870–301 500. One obtains 2 000 000 for titin

T-II and 560 000 for nebulin. Both proteins, in particular titin, are indeed extremely large.

DISCUSSION

Universal calibration

For isotropic random pore size distributions, theory predicts that the mean linear extension (L-measure) uniquely defines elution of all shapes [85], whereas viscosity radius does not [86]. Experimentally this is clearly not the case [23,24]. The majority of polymer types elute congruently in terms of their viscosity radii R_η, including the flexible and bent rod spectrin with spherical viruses in the present study (Table III). There are only two well established systems, schizophyllan and DNA, where the universal size parameter R_{SEC} seems to be larger than R_η but smaller than $L/2$ [23,28]. For DNA $R_{\mathrm{SEC}} \approx 1.15 R_\eta$ based on data from a single eluent. A recent claim that even proteins and pullulan do not co-elute [29] is at variance with numerous previous assessments regarding universal calibration of solid spheres and flexible chain polymers (reviewed in ref. 23), including work by the same group [28]. The admittedly unpleasant uncertainty regarding the principles of the SEC mecha-

nism cannot negate useful correlations of a more limited nature. Thus, comparison of solid spherical native proteins and viruses with nearly ideal flexible chains of the denatured proteins in terms of viscosity radii R_η is not nearly as problematic as generalization to all possible structures would be. Universal calibration in terms of viscosity radii R_η may be an empirical coincidence, but available theory also is insufficient: (1) real porous matrices are hardly ideal random and isotropic by the obvious need for ingress and egress of solutes; (2) pore walls are not plane but curved; (3) alignment of asymmetric units is energetically favoured; and (4) excess frictional drag may perturb chain–polymer configurations (a detailed account on the theory is in preparation).

Comparison of native proteins and viruses, which are more or less compact spheres, and of polypeptides denatured by GuHCl, which are charged random coils, may improve our perspectives of universal calibration, but matters are complicated. GuHCl is a chaotropic agent that reportedly affects the structure of gel matrices. Our data support earlier evidence from Eriksson and Hjertén [16] that cross-linked agaroses tend to change pore size on exposure to GuHCl. More remarkable is the reported congruent universal calibration on Sepharose, a different type of cross-linked agarose [18].

Horiike et al. [14] have previously established universal calibration of silicate TSK-SW with the GuHCl system and we have now extended this observation to TSK-PW. It therefore seems that universal calibration between solid spheres and charged coils holds. The deviation on Superose-6 then is explained by solvent-induced changes of the matrix itself. This does not contradict theory. Universal calibration may still hold within one given eluent. In fact, Superose yields reproducible GuHCl data that may be analysed directly but not compared with those for other solvents. Clearly, non-rigid gels may not be used for universal calibration amongst different eluents. The notion that uncharged polymer coils, such as pullulan, do deviate from solid spheres remains to be examined in more detail. For example, the intermediate case of proteins denatured in urea deserves critical observation.

Ionic strength dependence of elution

A surprisingly linear correlation between total apparent elution radius R and $I^{-1/2}$ has been observed previously for lower ionic strength in the case of repulsive interactions [23,25,26]. If this is extrapolated to high ionic strength, significant calibration differences are to be expected between $I = 100$ mM and 6 M GuHCl. That is, increasing salt would continue to shift calibration graphs to larger volumes, i.e., apparently smaller sizes. This is not the case. At high ionic strength the mentioned linearity in $I^{-1/2}$ breaks down and this has been verified experimentally for native conditions. It is also expected from theory. When the diffuse double layer shrinks below a certain size, other forces, such as hydration, take over to dominate the interfacial radius contribution R_{IF}. This effect might be enhanced by the fact that at increasing ionic strength the surface potential ψ_s may cease to be maximally saturated and constant but diminishes. The actual break point depends on the magnitude of the electrostatic interaction and thus some matrices may require 200 or even 500 mM salt to match 6 M GuHCl in a dual solvent experiment, whereas others may suffice with 60 mM salt.

Non-ideal effects

Polyelectrolyte adsorption is related to an effect known as hydrophobic interaction chromatography and, if it occurs, increases with increasing salt concentration. No retarded elution has been observed with 6 M GuHCl solutions, but a compensatory change of the TSK-PW matrix structure cannot be excluded in principle. Adsorption certainly is unable to explain the GuHCl data on Superose, nor can it explain the small ionic strength difference of TMV on TSK-6000PW under non-denaturing conditions.

Denaturant gradient chromatography

GuHCl gradient chromatography is used to study details of the denaturation of proteins [31–34]. A most interesting aspect of this work is a gradual shift towards smaller elution volumes, i.e., larger sizes, well before and possibly also after the sharp cooperative transition curve between the native and denatured conformation. This effect, which seems to be general, is best

demonstrated in the data for lysozyme [31] and ribonuclease [33]. Native proteins apparently enlarge their size in spite of retaining their native conformation, as judged by unchanged circular dichroism spectra. One obvious explanation is that the matrix changes its structure gradually to produce extreme points such as ones in Fig. 2 for Superose-6. Unfortunately, the workers involved did not perform a universal calibration to test this situation directly for their conditions. Convincing evidence for such a gradual shift was obtained, however, on TSK-SW [31,33], which presumably is resistant to a change in matrix structure [14]. Hence different explanations must be sought. The effect may be related to interfacial repulsion, i.e., the solvation shell enlarges with preferential adsorption of bulky guanidinium ions. This may partly compensate for residual ionic strength differences. This suggestion is amenable to testing by direct force methods [87,88] with moderately increasing amounts of GuHCl well before denaturation takes place.

Proteins in GuHCl

GuHCl dissociates all known protein aggregates and induces an almost ideal random coil structure to the monomeric polypeptide chain. The size of the coil is therefore directly related to the linear sequence length. Carbohydrates, introducing branch points, would then be expected to decrease the observed size, i.e., molecular masses calculated from eqn. 2 should attain an intermediate value between the total molecular mass and that for the plain polypeptide alone. However, carbohydrates do expand the chain and deviations from total molecular mass are only observed for high carbohydrate contents [9,11,16]. For the same reason, otherwise cross-linked chains may give erroneous results. Hence proteins need to be alkylated or studied under reducing conditions to break all disulphide bonds. Unreduced proteins in GuHCl have substantially smaller radii whose exact value is sequence dependent. As a representative case, consider bovine serum albumin whose unreduced GuHCl state is $[\eta] = 24.8$ ml g^{-1} instead of 50.1 ml g^{-1} for the reduced linear form [19]. Chemically cross-linked proteins are similarly expected to deviate. It was therefore very interesting to observe that our mildly cross-linked myosin preparation almost behaved like an ideal linear coil. Good results critically depend on the cross-linking conditions, however, and the procedure can hardly be recommended for general usage.

For small peptides the conventional calibration within the GuHCl system is obviously to be preferred. Using only one solvent throughout reduces the risks and uncertainties. Our analysis demonstrates that accuracy is better in a single solvent measurement. Actually, one may even calibrate directly in terms of molecular mass. For large peptides, where standards are rare or difficult to acquire, the procedure of universal calibration in two solvents on a selected rigid matrix is the method of choice. It should be mentioned that membrane proteins may also be studied if the lipid is first removed with acetone [89].

Other chaotropic agents

For chromatography GuHCl is currently the denaturant of choice to determine chain molecular masses, as denaturation by urea is frequently incomplete and the SDS–protein system supposedly violates universal calibration by eluting retarded (reviewed in ref. 23). A recent publication, however, insinuates that previous hydrodynamic constants, on which the latter judgment was based, are wrong [19]. Further studies of the SDS–protein system will be required. GuHCl chromatography also works with large polypeptides where SDS-PAGE fails (as with titin).

Accuracy of measurement

The accuracy of measurement has been reported for native proteins on Sephadex [90,91], Sepharose [92] and Sephacryl [92,93]. Differences between R_η and R_{SEC} are normally small and may indicate systematic bias due to interfacial effects. Large differences were found for membrane proteins in detergent solution on Sepharose [92], Sephacryl [92], Bio-Rad agarose [18], TSK-SW [94] and TSK-PW [94]. These figures, however, were based on R_s instead of R_η, which is not known in these cases, and the real bias may be comparable to those for other native proteins [23]. Generally, the detailed values depend on the method of calibration [93]

but the trend is typical for a given matrix. Different matrices may, however, exhibit different trends. Thyroglobulin, for example, has a similar R_{SEC} value on Superose-6 (Table V) ($R_{SEC} = 8.65$ nm) compared with $R_\eta = 8.6$ nm but a lower value on Sephacryl ($R_{SEC} = 7.93$ nm) [93]. Note, however, that this comparison may be biased by the reference standards studied in each instance. Similarly, ovalbumin has repeatedly been reported to elute prematurely ($R_{SEC} = 2.9$ nm on Sephadex [90] or even $R_{SEC} = 3.0$ nm [95]) compared with $R_\eta = 2.83$ nm. However, one report gives $R_{SEC} = 2.75$ nm on Sephacryl [93]. It is a challenging task for the future to single out and interpret systematic bias contained in this type of data. For now, let it suffice to say that errors in GuHCl and under native conditions are of comparable magnitude. As has been shown in the Results section, the accuracy of matching R_η by R_{SEC} is better than 5% in single solvent and better than 10% in dual solvent experiments.

Nebulin

Nebulin has not been isolated in native form. Most purification protocols used detergents such as SDS [38,46–48] or urea [49]. Final purification was mostly by SEC on Sephacryl S-500. The size of nebulin has been estimated so far only by SDS-PAGE and assigned values ranging from 500 000 to 900 000 [36,39,43,46,47]. The lack of well characterized standards in this size region is one reason for this large uncertainty. However, the size of nebulin is also species specific [46]. Here we describe a preparation method based on GuHCl and SEC. Nebulin is so large that all contaminants are easily removed. The subsequent analytical run on a different SEC matrix, TSK-6000PW, is straightforward and yielded a molecular mass of 560 000 for chicken pectoralis muscle. The most recent estimate by SDS-PAGE is 650 000 for this species. It is amongst the smallest types of nebulin [46].

Titin

Titin has been characterized by a variety of methods. Here we found a molecular mass for the monomeric polypeptide chain of titin T-II of 2 000 000 for the chicken type. Previously re-

ported molecular masses exhibit wide variations (Table I), which reflects methodological limitations. The main problem is the lack of adequate calibration standards. The discrepancy in the SDS-PAGE data is an artifact of myosin cross-linking and differences in size assignment for the cross-link ladder. The previous SEC study in GuHCl may have had similar problems unless it merely relied on extrapolation of the calibration graph with resulted in the same detrimental effect. Measurement of native titin T-II is complicated by aggregation, which was not recognized initially and lead to overestimates of the molecular mass. Sedimentation equilibrium studies in 6 M GuHCl, on the other hand, were conducted with a mixture of titin T-I and T-II, which again give values larger than expected for pure titin T-II. All things considered, our molecular mass of 2 000 000 for titin T-II is consistent with available information (Table I). Titin originating from either T-II$_A$ or T-II$_B$ eluted identically in 4 M GuHCl. Our molecular mass revises the mass per unit length of 2700 ± 900 nm^{-1} [45] to 2200 nm^{-1}.

An interesting and possibly important facet of the present study is the close similarity of titin T-II denatured in 6 M GuHCl with native titin T-II$_B$ in 600 mM salt. Here $R_\eta = R_{SEC} = 53$ nm, whereas native titin T-II$_B$ has been characterized on the same column as $R_\eta = R_{SEC} = 48$ nm (Table I) [42]. First, native titin T-II$_B$ is clearly a monomer. Using a molecular mass of 2 000 000 and s- value of 12.5 S with a partial specific volume of 0.727 yields $R_s = 38$ nm. The same value is calculated from the data of Maruyama et al. [39] for an aggregate mixture with a molecular mass of 2 700 000 and $s = 17$ S (see above). This gives strong support for our lower molecular mass value and demonstrates that Maruyama et al. studied a mixture of lateral aggregates. Early micrographs of titin molecules showed flexible, very long and heterogeneous strings [39–41]. Using improved specimen orientation methods before metal shadowing, titin molecules may be uniformly oriented [42], which facilitates length measurements but does not correspond to the structure in solution.

For proteins in GuHCl, experimental s values [58] may be analysed similarly to intrinsic vis-

cosities (eqn. 2) and the resulting correlation between diffusional Stokes radius (nm) and molecular mass is [62]

$$R_s = 2.32_0 \cdot 10^{-2} M^{0.511} \tag{3}$$

Hence one may calculate for $R_\eta = 53$ nm that titin T-II in 6 M GuHCl has a Stokes radius of $R_s = 38$ nm. In conclusion, native titin T-II$_B$ is highly flexible, close to a random coil and very similar in structure to its 6 M GuHCl counterpart. Native titin nonetheless has a defined secondary structure as judged by circular dichroism [96]. It contains β-strands but also "random" elements. In solution titin forms elastic coils that may be stretched out to 900-nm long rods during preparation for electron microscopy [42].

CONCLUSIONS

This study represents a practical application of chromatographic technique, but at the same time it maps features relevant to a mechanism of the SEC process which remains to be identified.

At high ionic strength calibration no longer changes with $I^{-1/2}$ and rather becomes congruent for various conditions. It is therefore possible to create a combined universal calibration for several different solvents, provided that the matrix is rigid and does not change in structure with changes in solvent. The latter has to be established for every case. If conditions of rigidity are fulfilled, native assemblies may be used to establish a high-molecular-mass calibration for 6 M GuHCl. In this manner it was possible for the first time to characterize two ultra-large polypeptides, titin and nebulin, by SEC in 6 M GuHCl. Their resultant molecular masses are 560 000 for nebulin and 2 000 000 for titin T-II. Native titin T-II monomers assume a conformation in solution that is very similar to that of their denatured counterpart. Error analysis demonstrates that a dual solvent approach, which was necessary in the present study, is inherently less accurate than studies using a single solvent. Whenever possible a single solvent design should be chosen.

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R. Nave et al. / J. Chromatogr. A 654 (1993) 229-246

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